AD_____

Award Number: DAMD17-98-1-8195

TITLE: Chromatin HMG-I(Y) as a Co-regulatory Protein for Estrogen Receptor Action in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Viroj Boonyaratanakornkit, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Denver, Colorado 80262

REPORT DATE: April 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2000	Annual Summary (1 Apr 99 - 31 Mar 00)

4. TITLE AND SUBTITLE
Chromatin HMG-I (Y) as a Co-regulatory Protein for Estrogen Receptor Action in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-98-1-8195

6. AUTHOR(S)
Viroj Boonyaratanakornkit, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
Denver, Colorado 80262

E-MAIL: viroj.boon@uchsc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

As sequence specific transcription factors, progesterone (PR) and estrogen receptor (ER) mediate the actions of progesterone and estrogen through direct activation of target genes. There is increasing evidence that PR and ER can also mediate non-genomic effects through activation of the Src/Ras/Erk signaling pathway. Using PR-A as the target in a yeast two hybrid screen, the SH3 domain of c-Cbl-associated protein (CAP) was identified to interact with the N-terminal domain of PR in a hormone dependent manner *in vivo*. PR was found to interact directly *in vitro* with the SH3 domain of CAP as well as selected SH3 domains of other signaling molecules including: c-Src and Hck. The N-domain of PR contains a sequence that conforms to a class II polyproline helix (PPII) ligand for SH3 domains and was shown by mutagenesis to be required for PR interaction with SH3 domains. Other nuclear receptors tested failed to interact with SH3 domains *in vitro*. ER enhanced PR-Src interaction and from PR-Src and ER tertiary complex. ER-Src interaction was mapped to Src SH2 domain. PR transiently and hormone-dependently associated with c-Src in T47D breast cancer cells and R5020 treatment caused a rapid and transient increased in c-Src kinase activity. *In vitro*, PR was a potent activator of tyrosine phosphorylated down regulated Hck via displacement of the intramolecular association of SH3 domain with the catalytic domain. These results suggest a novel function role for ER and PR as an activator of Src-kinase signaling pathways. Together, these findings may begin to unravel the molecular mechanism for non-genomic actions of estrogen and progesterone in breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	35
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

X Where copyrighted material is quoted, permission has been obtained to use such material.

X Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Vitaly Borovjagin 4/28/00

PI - Signature Date

Table of Contents

Summary of 1999-2000 research

Part I Chromatin HMG-I(Y) as a Co-regulatory Protein for Estrogen Receptor Action in Breast Cancer Cells

Through sequence analysis of several estrogen responsive gene promoters, we found that many of estrogen response elements (EREs) are composite elements for estrogen receptor (ER) and adjacent or overlapping sites for the non-histone chromatin protein HMG-I(Y). We, therefore, propose that depending on the context of the target gene promoter, HMG-I(Y) may either enhance or blunt the activity of ER and could contribute to cellular resistance to estrogen and antiestrogen frequently observed in breast tumors that express a functional ER. In the initial studies, we used gel-shift analysis to evaluate the effects of HMG-I(Y) on ER DNA binding. Two estrogen responsive gene promoter, human C3 and rat prolactin which contain ERE with putative HMG-I(Y) binding sites flanking or overlapping the ERE, were used as a model. Oligonucleotides containing ERE and flanking sequences from both promoters were used in gel-shift analyses. A synthetic ERE containing oligonucleotide, which has no putative HMG-I(Y) binding sites, was used as control. HMG-I(Y) binds very efficiently to both oligonucleotides containing ERE from C3 and prolactin promoter but did not bind to the synthetic ERE oligonucleotide. The binding of HMG-I(Y) to these DNA prevented ER from binding to the ERE whereas HMG-I(Y) had very little effects on ER binding to synthetic ERE oligonucleotides suggesting that the presence of HMG-I(Y) binding sites is important for HMG-I(Y) to affect ER DNA binding.

Since HMG-I(Y) inhibited ER DNA binding to oligonuleotide containing ERE from human C3 promoter, we next determined whether HMG-I(Y) affected ER transactivation of human C3 promoter using transient transfection. Hela cells were transfected with a reporter plasmid containing the same C3 ERE and flanking sequences used in gel-shift assays, various concentrations of HMG-I(Y), and ER expression plasmids. Cells were also co-transfected with expression vector for ß-galactosidase as an internal control to monitor transfection efficiency. Twenty-four hours after transfection, cells were treated with 10 nM estradiol or vehicle control. Forty-eight hours after transfection, cells were harvested and extracts were assayed for luciferase and ß-gal activity as described (2). HMG-I(Y) failed to affect C3 promoter ER dependent gene transcription at all concentration tested whereas in the same experiment co-expression of steroid receptor coactivator-1 (SRC-1), enhanced ER dependent gene transcription. In addition to Hela cells, HMG-I(Y) was also failed to affect ER dependent gene transcription in COS-1 and CHO-K1 cells.

Therefore, it is questionable whether HMG-I(Y) could have any effects on ER-dependent gene transcription even on promoter containing HMG-I(Y) binding sites overlapping the ERE as in human C3 promoter. In the original grant proposal, I purposed to biochemically characterized ER and HMG-I(Y) binding to EREs with overlapping or flanking HMG-I(Y) binding sites (Aim 2) and to use mutation analyses and cell transfection assay to assess the effects of HMG-I(Y) on ER-dependent gene transcription (Aim 3). Since I have been unable to demonstrate a biological

significance of HMG-I(Y) on ER-dependent gene transcription in whole cells, both my mentor and I agreed that it would be better for my training and career to discontinue the essentially negative results obtained with HMG-I(Y) and altered the specific aims to a related estrogen and progesterone receptor problems that has generated exciting preliminary results. The data for this new aim was generated before the beginning of the DOD breast cancer postdoctoral fellow award. The results and training gained from these studies is presented in the next section.

Part II Molecular Mechanism of Non-genomic Actions of Estrogen and Progesterone in Breast Cancer Cells

Introduction

Progesterone and estrogen regulate cell differentiation and proliferation in variety of cell types including breast cancer cells. The biological actions of these steroid hormones are though to be mediated primarily through progesterone and estrogen receptors (PR and ER) acting as transcription factors to directly regulate gene transcription. However, there is mounting evidence that, in addition to acting as transcription factors, steroid hormone receptors (SR) have rapid non-genomic action in cytoplasm and cell membrane that does not involve the transcriptional properties (see review ref (23)) . In breast cancer cells, treatment of estrogen and/or progesterone can rapidly stimulate c-Src, activate Src/$p21^{ras}$/Erk pathway, trigger DNA synthesis, and increase cell proliferation (14, 15). These non-genomic effects of estrogen and progesterone require ER and PR and are inhibited by both anti-estrogen (ICI 182780) and anti-progestin (RU486) (15). Recent study demonstrated that inhibition of Src/Ras/Erk pathway by microinjection of a dominant negative c-Src plasmid or treatment with MEK1 inhibitor, PD98059 inhibits the estrogen/progesterone-stimulated DNA synthesis of breast cancer cells, suggesting that non-genomic actions of ER/PR to activate c-Src mitogenic signaling pathway contribute significantly to growth and proliferative effects (5).

Activation of Src/Ras/Erk pathway has conventionally linked to activation by growth factors and causes cell proliferation, differentiation (4, 6, 9, 12). In addition, recent studies also suggested new roles for Src kinases in control of cell survival and angiogeneis (7, 32, 35). Activated Src-kinase has been shown in numbers of human breast cancer and colon carcinomas (18, 26) and has been implicated in tumorigenesis of the mammary epithelium (18). In addition, overexpression of Src in transgenic mice resulted in induction of mammary tumors (8). Thus, in mammary gland cells, estrogen and progesterone can simultaneously activate both genomic and non-genomic actions. Since mammary glands are one of the primary targets for both estrogen and progesterone and both hormone can rapidly activate Src/Ras/Erk pathway, understanding the molecular mechanism of crossed-talk between steroid hormone receptors (ER/PR) and Src will be essential for the understanding of breast cancer progression and treatment. However, very little is known about the molecular mechanism of how ER or PR activates Src-kinase activity. Therefore, the main purpose of

this study is to elucidate the molecular mechanism of non-genomic actions of estrogen and progesterone in breast cancer cells with major focus on estrogen and progesterone effects on c-Src signal transduction pathway. Better understanding of these non-genomic actions of estrogen and progesterone could provide us with new insights into how these steroid hormone act on cell proliferation and new therapeutic approaches to inhibit tumor growth and treatment of breast cancer.

In this study, we were searching for unique proteins, which interact with the N-terminus of human PR. Human PR is expressed as two proteins from a single gene by alternate use of two promoters; full length PR-B and truncated PR-A that is lacking the N-terminal most 164 aa (10). Thus the two proteins have identical sequence in the ligand and DNA binding domains and share about 2/3 of the same N domain sequence. Although the two PR proteins have essentially the same steroid and DNA binding properties, they exhibit distinct transcriptional activities. PR-B is generally a much stronger activator than PR-A. In certain cell and target promoter contexts, PR-A has minimal activation function and is capable of acting as a transdominant repressor of other steroid receptors (10, 13, 30, 31). Using the A form of PR as a target in yeast two hybrid screening, we isolated the SH3 domain of the c-Cbl associated protein CAP as a protein that interacts with the N-domain both *in vivo* and directly *in vitro.* This unexpected interaction lead to the discovery that the N-terminus of PR contains a proline rich consensus SH3 recognition sequence that binds efficiently to SH3 domains of several signaling molecules including Src tyrosine kinases. The presence of the proline rich consensus sequence and binding to SH3 appears to be a unique property of PR that was not detected in other members of the nuclear receptor family. In addition, the interaction between PR and Src, in vitro, was enhanced by addition of ER and a tertiary complex between ER-Src-and PR was also detected. *In vitro*, PR-SH3 domain interaction is capable of activating down regulated tyrosine kinase activity through displacement of an autoinhibitory intramolecular association of SH3 with other regions of the kinase. In mammalian cells, progesterone and PR activates Src kinases independent of receptor-dependent regulation of gene transcription. These results taken together suggest a novel non-genomic role for ER and PR as an activator of Src-tyrosine kinases through direct interactions with SH3 and SH2 domains.

Results

Yeast-two hybrid isolation of the SH3 domain of c-Cbl-associated protein (CAP) as a PR interacting protein.

To identify proteins that interact with the amino terminal domain of steroid receptors, a human U2-OS osteosarcoma cDNA expression library fused to the activation domain of GAL4 was screened by yeast two hybrid assay using human PR-A fused to the GAL4 DNA binding domain (GALDBD) as the bait. PR-A was chosen as the target because full length PR-B and the amino terminal domain alone fused to GALDBD exhibit high background activity as single hybrid proteins. In contrast, GALDBD/PR-A has no activity in the absence or presence of hormone (synthetic progestin R5020), but is capable of stimulating growth in His minus medium and the Lac Z reporter in response to R5020 in yeast transformed with the known PR interacting steroid receptor coactivator protein 1 (SRC-1) fused to the GAL4 activation domain (data not shown). Although PR-A is missing 164 N-terminal aa, it shares the remaining approximately 2/3 of the N-domain with full length PR-B and thus has the potential to isolate novel proteins that interact with the region of the N domain that is common to the two PR isoforms. Six colonies were isolated that interacted with GALDBD/PR-A in a manner dependent on the presence of both PR-A and the isolated cDNA inserts from the library. Two of the colonies contained identical sequences and the other four contained unique sequences. Analysis of the GenBank data base revealed that one of the unique colonies encodes for a polypeptide with 72% amino acid sequence homology to the carboxyl terminal Src homology 3 (SH3) domain of the mouse c-Cbl-associated protein (CAP) and thus is likely the human homologue of mouse CAP (mCAP). CAP was originally isolated from a mouse 3T3-L1 adipocyte cDNA expression library by yeast two hybrid assay using full-length c-Cbl as the target (25). CAP contains three adjacent SH3 domains in the C-terminus and an N terminal region closely related in sequence to the peptide hormone sorbin. The open reading frame of the human cDNA obtained from our screening of the U2-OS library corresponds to the C-terminal most SH3 domain of mouse CAP, termed SH3-C. The functional role of CAP is not well defined. It directly binds to c-Cbl and the insulin receptor and is thought to function in insulin signaling and in trafficking of signaling molecules. It also interacts with the focal adhesion kinase, p125FAK and appears to have a role in signaling pathways that stimulate formation of actin stress fibers and focal adhesions (24).

PR interaction with CAPSH3 domain is hormone-dependent *in vivo* by yeast two hybrid assays.

To broadly map the region of PR-A that interacts with CAPSH3 domain and to determine whether the interaction is hormone dependent, the CG1945 yeast strain was transformed with the C-terminal ligand binding domain of PR (hLBD aa 633-933) fused to GAL4DBD or GAL4-PR-A along with CAPSH3/ AD. Transformants were grown on plates with R5020 or vehicle (ethanol) and β–galactosidase was measured in cell lysates by a liquid assay . As shown in Figure 1A, no significant induction of β-gal over the single hybrid controls was mediated by the C-terminal LBD of PR, either in the absence or presence of R5020. In contrast, GAL4PR-A mediated a significant induction of β-gal over the single hybrid control that was stimulated by R5020 (Fig. 1). A weak interaction between PR-A and CAP3HS domain was observed in the absence of hormone, which was further stimulated another 5 fold in the presence of R5020. Thus, in yeast interaction with CAP-SH3-C is strongly stimulated by R5020 and requires either the DNA binding domain or the N-terminal domain of PR.

Direct binding to CAPSH3 domain *in vitro* is dependent on proline rich sequences in the N terminal domain of PR.

To determine whether PR interactions with CAP-SH3 domain observed in yeast and mammalian two hybrid assays are due to a direct association between the two proteins, pull down experiments were performed with recombinant mCAP-SH3 domain expressed as a GST fusion protein (25) and PR-A or PR-B expressed as polyhistidine tagged proteins in the baculovirus expression system. GST alone, as a control for non-specific binding of PR, and GST-mCAP-SH3-C were immobilized to glutathione Sepharose beads and incubated with PR-A or PR-B prepared as whole cell extracts from Sf9 insect cells. To determine the influence of hormone on PR-CAPSH3 domain interactions *in vitro*, PR was either bound to R5020 during expression in SF9 cells or was left unbound to hormone. As shown in Fig 2B, both PR-B and PR-A interacted efficiently and specifically to immobilized GST-mCAP-SH3C. However, unlike the results obtained in yeast and mammalian two hybrid assays, interaction between PR and CAPSH3 *in vitro* was not hormone dependent. A comparable interaction with CAPSH3 *in vitro* was observed with PR in the absence of ligand or when bound to R5020 (Fig 2B).

To determine the region of PR required for *in vitro* interaction with CAPSH3, various fragments of PR were expressed as polyhistidine tagged proteins in the baculovirus system and analyzed for their ability to interact with GST-CAPSH3 by pull down assay. Consistent with yeast two hybrid results, the LBD of PR (bound to R5020) did not interact with CAPSH3, nor did the DNA binding domain of PR (Fig 2C). However, the N-terminal domains of PR-A or PR-B linked to

the DBD (AN DBD and BN DBD) both interacted with CAPSH3 as well as the interaction with full length PR-A and PR-B. These results indicate that the CAPSH3 interaction site is located within the N-terminal domain region (aa 165 - 552) that is common to both the A and B isoforms of PR.

SH3 domains recognize proline-rich sequences that adopt polyproline II helix conformation (17). Human PR contains a proline-rich sequence in the amino terminal domain between aa 421-428 (PPPPLPPR) that conforms to class II peptide ligands for SH3 domains that contain the core sequence XPPLXR (X= any aa). To determine whether this sequence in PR is required for CAPSH3-C binding, three prolines (P422A, P423A, and P426A) were converted to alanines by site directed mutagenesis to yield the sequence PAAPLAPR (Fig 2A). This proline mutant in the context of full length PR-B (PR-B_{mPro}) was cloned into a baculovirus vector with a polyhistidine tag and expressed in Sf9 insect cells and analyzed for binding to CAP-SH3 by GST pull down. As compared with baculovirus expressed wild type PR-B which binds efficiently to GST-CAP-SH3C, the PR-B_{mPro} did not interact (Fig 2D), indicating that the proline rich sequence between aa 421 and 428 is the interaction site for CAPSH3C domain.

Other nuclear hormone receptor do not interact with CAP-SH3 domain

An examination of the amino acid sequence of other members of the nuclear hormone receptor superfamily revealed that only PR contains this proline-rich consensus sequence for polyproline II helix peptides, suggesting that SH3 domain interaction is a specific property of PR that does not extend to other nuclear receptors. To directly test whether CAPSH3 interaction is unique for PR, the ability of several other nuclear receptors to bind to CAPSH3 *in vitro* was analyzed by GST-pull down assay. Full length human estrogen receptor (ER), glucocorticoid receptor (GR), androgen receptor (AR) and thyroid hormone receptor-beta (TR–β) were expressed in baculovirus as polyhistidine tagged proteins and analyzed for binding to CAP-SH3-C-GST fusion protein by pull down assay as described above. As compared with human PR-A used in this experiments as a positive control, no interaction was observed between CAPSH3 and any of the other nuclear hormone receptors tested (Fig 3). These results indicate that PR is the only steroid class of receptor, and perhaps the only nuclear hormone receptor that has the ability to bind to SH3 domains.

PR interacts with SH3 domain of other proteins

The fact that PR contains a consensus proline-rich class II (PPII) sequence that serves as a ligand for SH3 domains, suggests that PR may bind to SH3 domains in other signaling molecules. Therefore we analyzed the ability of PR to interact *in vitro* by GST-pull down assays with SH3 domains of several other proteins. Baculovirus expressed PR-B (bound to R5020) was incubated with GST fusion protein-SH3 domains of Hck, Fyn, p85, Grb, Crk,

Src, Cortactin, Cas, Nck, the other two SH3 domains of CAP (A and B) and the C-terminal SH3 domain of CAP(C) as a positive control. Protein complexes immobilized to glutathione-Sepharose beads were eluted and PR-B was detected by immunoblotting with the PR specific MAb 1294. In addition to CAP-SH3C domain, PR interacted strongly with SH3 domains of Hck, p85, Grb and c-Src, weekly with SH3 domains of Fyn and Crk, and failed to interact with the other two SH3 domains (A and B) of CAP, Cortactin, Cas, and Nck (Figure 4, upper panel). Thus, PR interacts with for only a subset of SH3 domain proteins, including interaction with only one SH3 domain (SH3-C) of the three present in CAP. To test whether interactions between PR and other SH3 domains require the proline rich sequence of PR between aa 421-428, the baculovirus expressed proline mutant, PR-B_{mPro}, was analyzed in GST-pull down assays for its ability to interact with these same SH3 domain-GST fusion proteins. As shown in Figure 4 (lower panel), no interaction was observed between PRB_{mPro} and all tested SH3 domains demonstrating that, like CAPSH3-C, the interaction between PR and other SH3 domains requires the PPPPLPPR sequence present in the N-terminus of PR.

Direct binding of ER and PR to Src tyrosine kinase

To determine whether SH3 domain alone is sufficient for PR interaction with full-length c-Src and whether there might be other interacting sites, we tested the ability of PR-B to interact with different domain of c-Src (Fig 5A) in GST-pull down assays. Baculovirus expressed purified PRB was incubated with GST fusion protein of c-Src domain, including the N-terminus unique domain (U), SH3, SH2 or the combination of U-SH3-SH2, and SH3-SH2. Bound proteins immobilized to Glutathione-Sepharose beads were eluted and analyzed by immunoblotting with PR specific Mab 1294. In addition to the strong SH3 interaction of PR, a weak interaction of PR with SH2 was detected (Figure 5B) that was mapped to the DBD. Interestingly, PR-interaction with Src SH2 domain was increased using crude extract (data not shown)

In addition to progesterone, treatment of estrogen stimulates Src kinase activity in breast cancer cells( ). We next tested the ability of human ER to interact with c-Src. Baculovirus expressed purified ER was incubated with GST-fusion protein of c-Src domain and analyzed as described above. Purified ER interacted only with the SH2 domain of c-Src (Fig 6). No interaction was observed with the U and SH3 domain. Since the presence of ER was important for progesterone to activate c-Src (15), we next investigated whether ER affected PR-Src interaction. Baculovirus expressed purified PRB, or PRB_{mPro}, was incubated with GST fusion protein of c-Src U-SH3-SH2-domain in the presence or absence of purified ER. The addition of ER enhanced PRB-Src interaction but had no effect on Src interaction with PRB_{mPro}. These results suggest that c-Src through its SH2 and SH3 domain interact with ER and PR, respectively, and can form a stable tertiary complex with ER and PR, *in vitro*.

Progesterone agonist (R5020) induces transient association of PR with c-Src and stimulates c-Src activity in breast cancer cells

We next determined if the interaction between PR and c-Src could be detected in breast cancer cells. T47D breast cancer cells, which contain ER and express constitutively high levels of PR, were treated with 10 nM of R5020 for 2, 5 or 7 min. Cells were lysed and immunoprecipitated with Src specific MAb 327 or control antibodies. Proteins from immunoprecipitates were immunoblotted with PR specific MAb 1294 (Figure 7A). A transient hormone-dependent association between PR and c-Src was consistently observed at 5 min after hormone treatment. To determine if treatment of R5020 affected c-Src activity, cell lysates were immunoprecipitated with Src MAb (327) and assayed for c-Src activities using acid-modified enolase as substrate (14). As shown in figure 7B, Src kinase activity was stimulated as early as 2 and 5 min after hormone treatment and decreased thereafter.

Since we demonstrated that the proline rich PPII sequence was required for PR-Src interaction *in vitro,* we next tested if PRB_{mpro}, could support Src kinase activation upon progesterone treatment. Cos-7 cells were transfected with expression vectors for c-Src, and human ER along with wild type PRB or mutant PRB (PRB_{mpro}). Forty-eight hours after transfection, cells were left untreated or treated with 10 nM R5020 for 5 min. Cells were then lysed, immunoprecipitate with 327 MAb and assayed for c-Src activity using enolase as substrate. c-Src activity in cells expressing wild-type PRB was stimulated after 5 min of R5020 treatment, however, c-Src activity form cells expressing PRB_{mpro} was unaffected (Figure 7C). These results suggest that PP II helix sequences are not only required for binding to SH3 domain of c-Src but also required for PR-mediated c-Src activation.

Activation of the Src-family tyrosine kinase Hck by PR

Members of the Src-family tyrosine kinases possess a variable region followed by SH3 and SH2 domains, the catalytic domain, and a short carboxyl terminal tail. The SH2 and SH3 domains play an important role in regulating tyrosine kinase activity (28, 33, 34). In addition to mediating protein-protein interaction with other signaling molecules and enabling the kinase to phosphorylate specific cellular substrates, the SH2 and SH3 domains are involved in an autoinhibition mechanisms through intramolecular protein interactions that resemble the peptide recognition ligands for these domains. The SH2 domain interacts with tyrosine 527 in the C-terminal tail while the SH3 domain interacts with the linker region between the SH2 and catalytic domain that forms a left handed polyproline like helix. These intramolecular interactions dock the SH2 and SH3 domains against the catalytic domain and maintain the enzyme in a closed inactive conformation (Fig 8A). Conversion to the open catalytically active conformation can be achieved by dephosphorylation of tyrosine 527 and displacement of the SH2 interaction with the C-terminal tail, or by displacement of the SH3

interaction with the linker region by competition for binding with an external SH3 peptide ligand without the need for dephosphorylation (16, 20). The biological significance of these SH2 and SH3 interactions in controlling Src kinase activity has recently been demonstrated *in vivo*. Mutation of SH2-kinase linker preventing binding to the SH3 domain of Hck converted wild type to constitutively active Hck and demonstrated strong transforming activity in rat fibroblast cells (3).

Since PR binds efficiently to the SH3 domains of both Src and Hck (hemopoietic cell kinase) in a manner dependent on the proline rich sequence in its N-terminus and both Src and Hck have a similar closed-conformation structure as determine by X-ray crystallography (28, 33), we next investigated whether PR can activate down-regulated Hck through displacement of intramolecular SH3 interactions (This section of the study was done in colloboration with Dr. W.T. Miller at State University of New York at Stony Brook, NY.) Hck was co-expressed with C-terminal Src kinase (Csk) in Sf9 cells to produced the enzymatically down-regulated closed conformation of Hck. . The activation of down-regulated Hck was measured by the phosphocellulose paper assay (16). Baculovirus expressed purified polyhistidine-tagged PR-B was pre-incubated with purified down-regulated Hck (16) for 10 min before addition of enzyme substrate. After the addition of peptide substrate, the reactions were carried out for 10 min in the presence of γ-^{32}P-ATP. The Hck activity was determined by the amount of ^{32}P-incorporation into the peptide. Addition of wild type PR-B (bound to R5020) caused a 5 to 6 fold activation of down-regulated Hck in a PR dose -dependent manner (Fig 8B). To further map the amino acid sequences required for activation of down regulated Hck, 14-mer peptides with sequences correspond to the polyproine sequences in wild type (PLGPPPPLPPRATP) or mutant PR-B (PLGPAAPLAPRATP) were synthesized. These peptides were then tested for their ability to activate down-regulated Hck. Wild-type peptides were efficiently activated down-regulated Hck (5 to 6 fold activation), however, mutant peptide had no effect (not shown). Together, these results suggested that PR through its proline rich sequences bind and activated Hck and that the proline rich PPII sequences are the minimum determinant in PR required to activated down-regulated Hck.

Discussion and Future Directions

Using yeast two-hybrid screening, we found that human PRB interact with SH3 domain of CAP and the interaction sites was mapped to the N-terminus of PR. The interaction was hormone dependent in yeast, but hormone independent *in vitro*. We mapped PR-SH3 interaction site to the proline rich region of PR (aa 421-428) which conforms to polyproline type II helix, a consensus sequence for SH3 ligand. Mutation of these sequences abolished the interaction. The interaction with CAP-SH3c domain seemed to be a unique property of PR, no other nuclear receptors tested interacted with CAP-SH3C domain. In addition to CAP-SH3, PR also directly interacted with SH3 domains of several signaling

molecules including c-Src and Hck. Using co-immunoprecipitation, we demonstrated that PR transiently and hormone-dependently associated with c-Src in T47D breast cancer cells and R5020 treatment caused a rapid and transient increased in c-Src kinase activity. Consistent with our *in vitro* binding data, the proline-rich sequence in the N-terminus of PR is required to activated c-Src. Finally, we showed that PR activated down-regulated Hck through SH3 domain displacement and further demonstrated that the proline rich sequence is the minimum determinant in PR required to activated down-regulated Hck.

Although we are now starting to learn more about the molecular basis for PR and c-Src interaction and mechanisms for cross-talked between nuclear receptor (ER and PR) and Src/Ras/Erk signaling pathway, there are still several unanswered questions. The interaction between PR and SH3 domain is hormone-dependent only *in vivo* (in yeast and in mammalian cells) and is hormone independent *in vitro*. There are several explanations for this difference. One of the most likely explanations is that, in the cells, PR as an unliganded receptor interacts with a set of proteins, which could interact with the proline rich region, and block PR from interacts with SH3 domain. Upon ligand binding and conformational change, the proline rich region of PR is now free to interact with SH3 domain. In our *in vitro* binding assays, the proline rich region may not be blocked in the unliganded receptor and, therefore, allow the unliganded receptor to interact with SH3 domain.

PR is largely a nuclear protein whereas c-Src is largely a cytoplasmic protein. This raised the question whether PR and Src exist in the same cell compartment. Despite the fact that both proteins usually reside in different compartment within the cell, both proteins are shuttling between cytoplasm and nucleus, therefore, it is possible that some subpopulation of both proteins be in the same compartment at one time and allow the interaction to occur. In addition, in this study, we showed that only a small fraction of PR transiently and hormone-dependently interacted with c-Src. However, this may be significant since we also observed progesterone activation of Src kinase activity at the same time and only with wild type PRB but not PRB_{mpro}. There are some evidences of membrane-associated nuclear receptor, which would also allow the interaction with c-Src (1, 19). One possibility is that PR activation of c-Src occurs at certain normal developmental stages and may play a role in breast tumorigenesis. During proliferative stages of early mammary gland development, PR is predominantly localized in the cytoplasm (29). In addition, a recent study on the progression of endometrium cancer showed that in normal proliferative and hyperplastic endometria, PR is localized to the nucleus and retains nuclear localization during the progression from well-to-poorly differentiated adenocarcinoma and, at these stages, progesterone treatment inhibit cell proliferation. However, with the progression to endometrium cancer, PR is localized to the cytoplasm and these cancer cells are no longer response to growth inhibitory effect of progesterone (11).

We also found that ER interacted with the SH2 domain of Src and enhanced PR-Src interaction, *in vitro*. In addition, ER appeared to enhance the ability of PR to activate c- Src. In Cos-7 transfection experiments, PR in the absence of ER was not sufficient to activate c-Src (data not shown). The role of ER in PR-Src interaction and activation of c-Src remains unclear. We are now in the process of mapping domains of ER required for interaction with SH2 domain of c-Src and determining how these ER interactions affects c-Src activation. As a first step, we have successfully expressed the DNA binding domain and ligand binding domain of human ER in bacteria and will be using these domains and c-Src-SH2 domains in pull-down assays. To further characterize the interactions in whole cells, we are now developing a mammalian two-hybrid assay to detect PR and Src interaction and will determine how ER affects this interaction in mammalian cells.

In this study, we demonstrated that PR effectively activated down-regulated Hck. According to our data, we proposed a model for the mechanism by which ER and PR activates Src-kinase activity (Figure 9). In this model, the proline-rich sequence in the N-terminal of PR serves as a ligand for SH3 domain and disrupts the interaction between the linker region and SH3 domain of Src. This binding allows Src molecule to open-up and causes tyrosine phosphorylation at position 420 keeping Src in an active conformation. Binding to the Src-SH2 domain and disruption of the interaction with phosphotyrosine 527 can also activate Src-kinase activity. It is still unclear whether interactions between PR/ER with Src-SH2 domain are sufficient to activate Src-kinase activity. Interestingly, our preliminary data using purified c-Src and purified PRB showed that c-Src phosphorylated PRB (data not shown). Since c-Src phosphorylates tyrosine residues and SH2 domain binds to phosphotyrosine peptides, it is conceivable that once PR activates Src, PR becomes a substrate for Src and itself becomes tyrosine phosphorylated. This tyrosine phosphorylated PR, in turn, binds to SH2 domains of Src and starting a new round of activation. In fact, recent study demonstrated the enhancement of Src kinase activity with substrates containing SH2 domain binding sites (21). We will investigate the contribution of Src- SH2 domain interaction with ER and PR in the activation of down-regulated Hck by using mutant down-regulated Hck which can no longer be activated via SH2 displacement (22). In wild-type down-regulated Hck, the activation can be achieved by displacing both SH2 and SH3 interaction. Using mutant PRB which can no longer activate through SH3 displacement or ER which does not interact with SH3 domain and by comparing activation obtain from wild-type and SH2-mutant down-regulated Hck, we will be able to assess the contribution of SH2 displacement by ER or PR in the activation of Hck kinase activity.

Experimental plans for the year 2000-2001

1) Mapping domains of ER required for c-Src SH2 domain interaction using *in vitro* binding assays (GST-pull down). These experiments will be carried out in the same fashion as mapping domains of PR for c-Src SH3 and SH2 interactions. Once the domain of ER required for this interaction is identified, this region of ER will be mutated and the effects of these mutations on ER interaction and activation of c-Src will be determined.
2) Mapping phosphotyrosine residues in ER and PR, which are phosphorylated by c-Src. In collaboration with Dr. N.L. Weigel at Baylor College of Medicine, Houston, TX, we will determine which tyrosine residues in ER and PR are phosphorylated by c-Src. These tyrosine residues will be mutated. The effects of these mutations in ER and PR on activation of Src/Ras/Erk pathway as well as activation of ER and PR dependent gene transcription will be assessed.
3) Determine how ER and PR interactions with the SH2 and SH3 domain of c-Src affect estrogen and progesterone-mediated breast cancer cell proliferation by using ER and PR mutants described in 1 and 2. ER and PR mutants, which prevent interaction with and fail to activate c-Src, will be tested to determine if these mutants affect estrogen- and progesterone-mediated breast cancer cell proliferation. T47D(Y) breast cancer cells (27), which no longer expressed PR and expressed very low levels of ER will be used as a model to study estrogen-, progesterone-mediated cell proliferation. Since these cells are derived from hormone responsive breast cancer cells, they are likely to have all the machinery required for hormone responses. Cells will be infected with adenovirus expressing wild type or mutant receptors and cell proliferation parameters (cell numbers, number of cells entering S-phase by Flow cytometry, DNA synthesis, and the ability to form colonies in soft agar) in response to estrogen and progesterone treatment will be determined.
4) Developing mammalian two-hybrid assays to demonstrate PR and c-Src interaction in mammalian cells and determine how ER affects PR-Src interaction.
5) Determine if ER-ß interact with c-Src and its effect of on c-Src activation. All experiments with ER so far were carried out with ER-∝, therefore, it is important to determine if ER-ß ,which binds similar ligands as ER-∝ but has different patterns of tissue expression, will have similar effect on c-Src activation. (Using baculovirus expression system, we had successfully expressed and purified 6x histidine-tagged human ER-ß)
6) Developing baculovirus expression system for down-regulated c-Src. Since all *in vitro* Src-kinase activation experiments presented in this study were carried out with down-regulated Hck, it is, therefore, important to confirm these findings down-regulated c-Src. Although Hck and c-Src have similar molecular structure, Hck is expressed only in cells of hemopoietic origin. Since c-Src, but not Hck, expresses in mammary tissue and has

been shown to associate with breast cancer, studying the effects of PR and ER on activation of down-regulated c-Src will provide us with information more relevant to the biology breast cancer.

Summary

Key Research Accomplishments

- Human PR through its proline-rich sequence (aa 421-428) interacts with SH3 domains of several signaling molecules, including Src-kinase family : c-Src and Hck
- Mutation of three proline residues in the proline-rich sequence abolishes PR interaction with SH3 domain of c-Src.
- Interaction with SH3 domain appears to be a unique property of PR. No other nuclear receptors tested interacts with SH3 domain
- ER interact with SH2 domain of c-Src
- Progesterone treatment causes a rapid and transient interaction between PR and c-Src and rapidly stimulates c-Src kinase activity.
- PR activates down-regulated Hck by disrupting the interaction between the linker region and SH3 domain of Hck and displacing with the proline-rich sequence in the N-terminus and keep Hck in an open-active conformation.

Reportable Outcomes

Abstract for each presentation is included in Appendices

- Poster presentation at Keystone Symposia Nuclear Receptors 2000, March 25-31,2000, Stemboat Springs, Colorado. Abstract #208
- Oral presentation at The Endocrine Society's 82nd Annual Meeting,. June 21-24, 2000, Toronto, Canada. Abstract # 1971
- The 2000 Burroughs Welcome Fund Travel Award from the Endocrine Society. A copy of the award letter is included in an appendix.
- Manuscript in preparation. Title " Progesterone receptor contains a proline rich sequence in the amino terminus that directly interacts with SH3 domains and activates Src family tyrosine kinases".

Conclusion

In this study, we had unexpectedly discovered a novel direct interaction between PR and SH3 domain of c-Src. Through this interaction PR can directly activate c-Src by SH3

domain displacement. This unorthodox interaction between PR, a steroid hormone receptor, and c-Src, a signaling molecule, could help explain the molecular basis for cross-talked between steroid hormone receptor and peptide growth factor signaling pathway. With these new finding, we may now begin to understand the molecular basis for non-genomic actions of estrogen and progesterone.

Figures

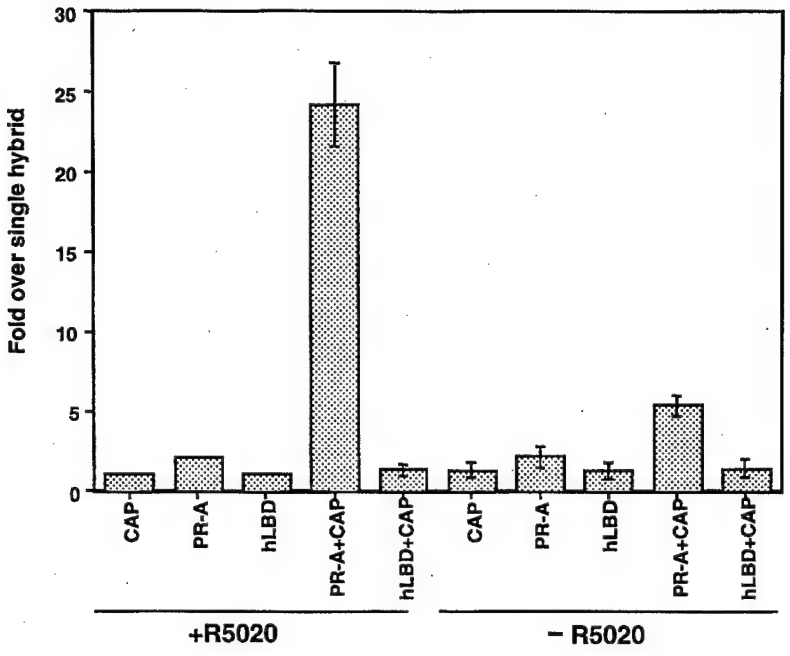
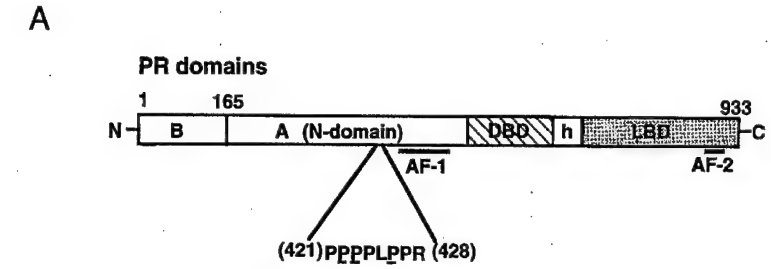
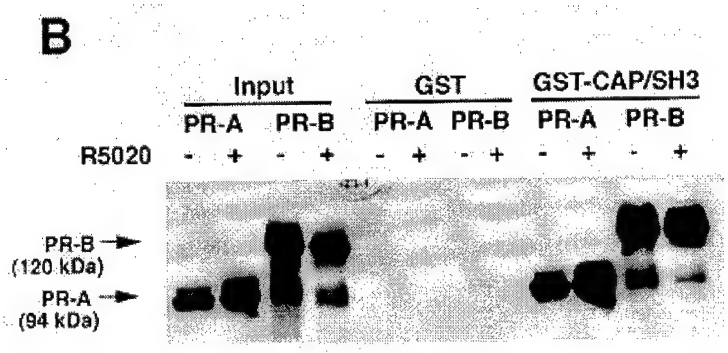
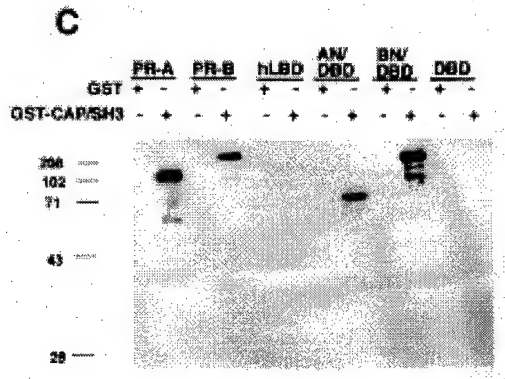
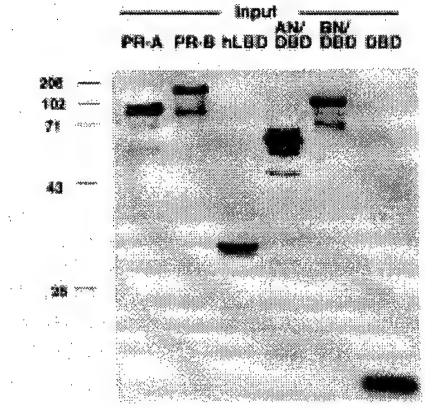


Figure 1. Hormone-dependent interaction of PR with CAPSH3 domain in yeast two-hybrid assays. PR-A or the hinge plus LBD domain of PR(633-933) fused to GAL4DBD were expressed as single hybrids or with CAPSH3 fused to the GAL4 activation domain in the yeast strain CG1945 that contains an integrated B galactosidase reporter bearing upstream GAL4 activator sequences. Transformed cells were grown in the presence of vehicle or R5020 (100nM). ß- galactosidase activities were measure based on equal input of cell lysate protein. The data are represented as fold hormone induction of enzyme activity normalized to OD600 mediated by the presence of two hybrids over the one hybrid controls and the values are averages (±SEM) from three independent determination


A
PR domains
1
165
933
N
B
A (N-domain)
DBD
h
LBD
C
AF-1
AF-2
(421)PPPPLPPR (428)


B
Input
GST
GST-CAP/SH3
PR-A
PR-B
PR-A
PR-B
PR-A
PR-B
R5020
- + - + - + - + - + - +
PR-B
(120 kDa)
PR-A
(94 kDa)


C
PR-A
PR-B
hLBD
ΔN/DBD
ΔN/ΔDBD
DBD
GST
GST-CAP/SH3
206
102
71
43
29


Input
PR-A
PR-B
hLBD
ΔN/DBD
ΔN/ΔDBD
DBD
206
102
71
43
29

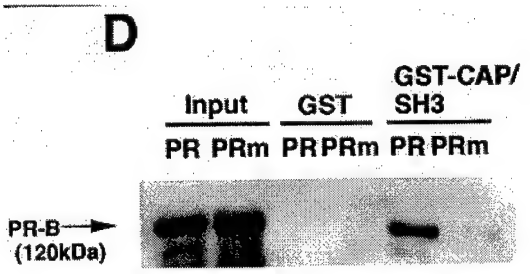

D
Input
GST
GST-CAP/
SH3
PR PRm PR PRm PR PRm
PR-B
(120kDa)

Figure 2. ***In vitro*** **interaction of PR with CAP-SH3 domain is hormone independent and requires the proline rich class II sequence in the amino terminal domain. A)** Schematic of human PR and the proline rich sequence between amino acids 421 to 428 that conforms to the consensus class II polyproline ligand for SH3 domains. The proline residues underlined were converted to alanines to create the PR mutant PR(mPro). The amino terminal domain of PR-A is missing the first 164 amino acids present in full length PR-B. LBD, ligand binding domain, DBD, DNA binding domain, h, hinge region, AF-1, transcriptional activation domain -1 and AF-2 transcriptional activation domain -2. **B)** Baculovirus expressed PR-A and PR-B in the absence of ligand or bound to the synthetic progestin R5020, were prepared as crude extracts for Sf9 cells and incubated with either GST or GST-CAPSH3 immobilized to glutathione Sepharose beads. PR bound to the beads was eluted and analyzed by immunoblotting with the PR specific monoclonal antibody PR 1294. The input lanes are immunoblots of 10% of Sf9 cell extracts incubated with GST or GST-CAPSH3 domain proteins. **C)** Upper panel. PR-A and PR-B plus the PR domain polypeptides indicated were expressed in baculovirus with 6 x polyhistidine tags; hLBD, hinge plus ligand binding domain; ANDBD, amino terminal domain of PR-A plus the DNA binding domain; BNDBD, the amino terminal domain of PR-B plus the DNA binding domain; DBD, DNA binding domain. Crude extracts of infected Sf9 cells were incubated with either GST or the GST-SH3 domain proteins immobilized to Glutathione Sepharose beads and the bound PR polypeptides were eluted and analyzed by immunoblotting with a monoclonal antibody specific to the 6 x polyhistidine tag (clone 1162/F1). PR-A, PR-B and the hLBD were bound to R5020. Bottom panel: Immunoblot of 10% of the input PR polypeptides as detected with a 6 x polyhistidine MAb. **D)** Wild type PR-B(PR) and mutant PR containing alanine substitutions of P421, P422 and P426 (PRmPro) were expressed from baculovirus vectors in Sf9 cells in the presence of R5020, incubated with either GST or GST-CAP/SH3 domain immobilized to Glutathione Sepharose and bound PR proteins were eluted and analyzed by immunoblotting with the PR specific MAb PR1294. Input PR and PR(mPro) represents an immunblot of 10% of the Sf9 extracts incubated with GST or GST-SH3.

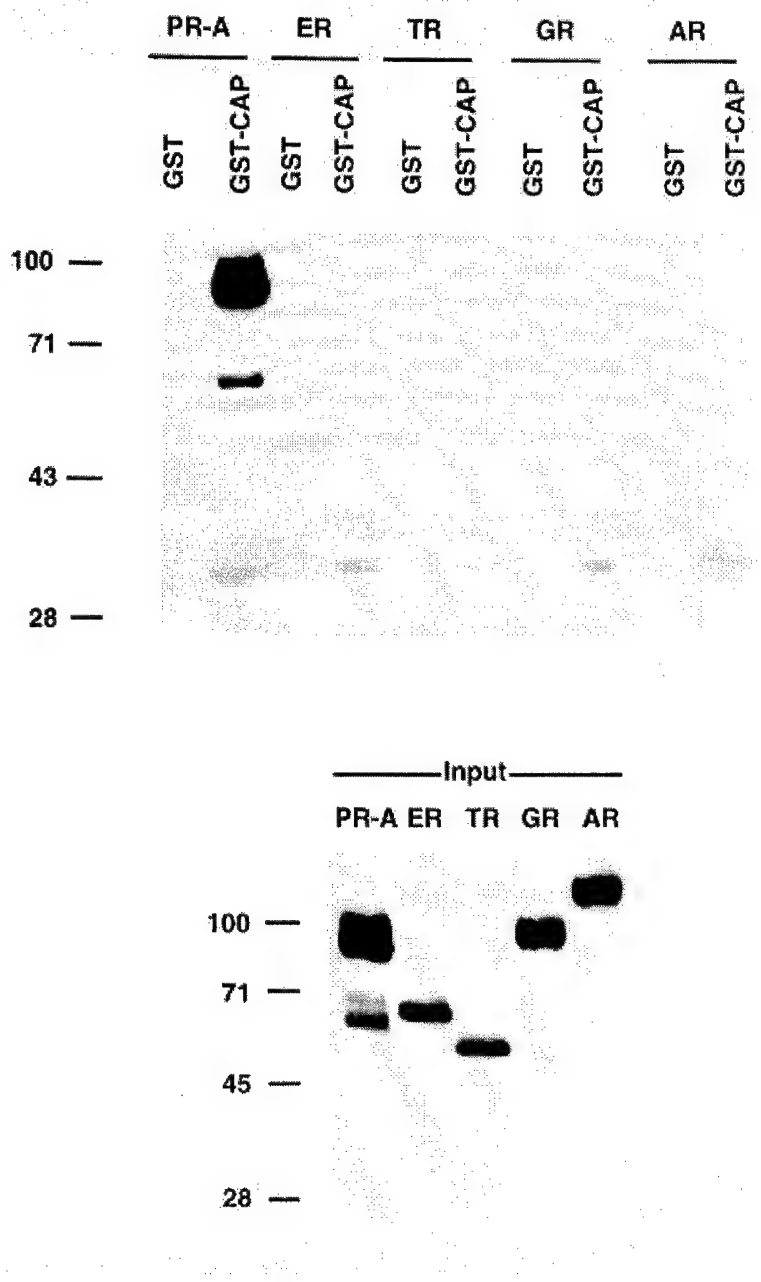


Figure 3. Other nuclear hormone receptors do not interact *in vitro* with CAPSH3 domain. Human PR-A, ER, TRβ, GR and AR were expressed in the baculovirus system with N terminal polyhistidine tags and incubated with their cognate hormone ligands in Sf9 cells. Whole cell extracts were incubated with GST or GST-CAPSH3 immobilized to Glutathione Sepharose beads and bound receptors were analyzed by immunoblotting with the MAb specific for the 6x polyhistidine tag (upper panel). The lower panel is an immunoblot of 10% of the whole cell extract input for each receptor using the MAb specific to 6 x polyhistidine tag.

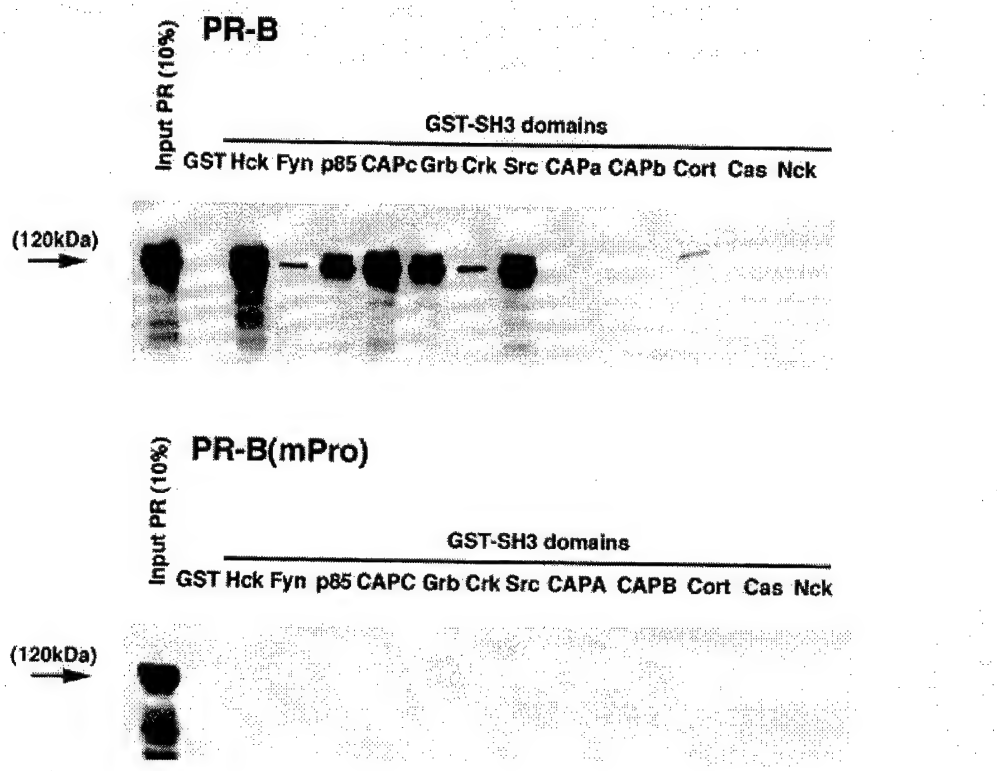
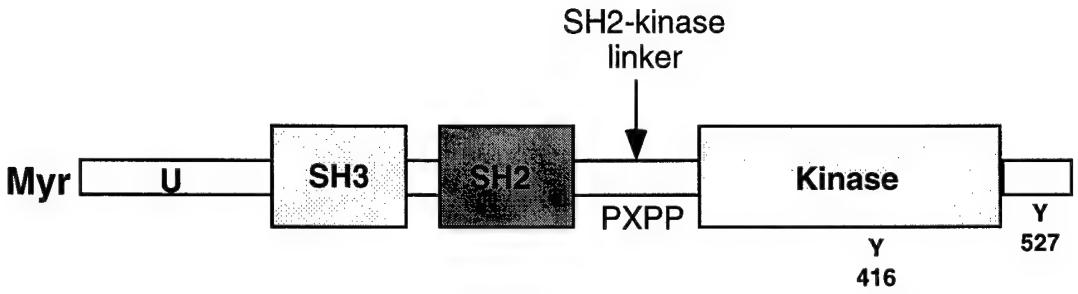
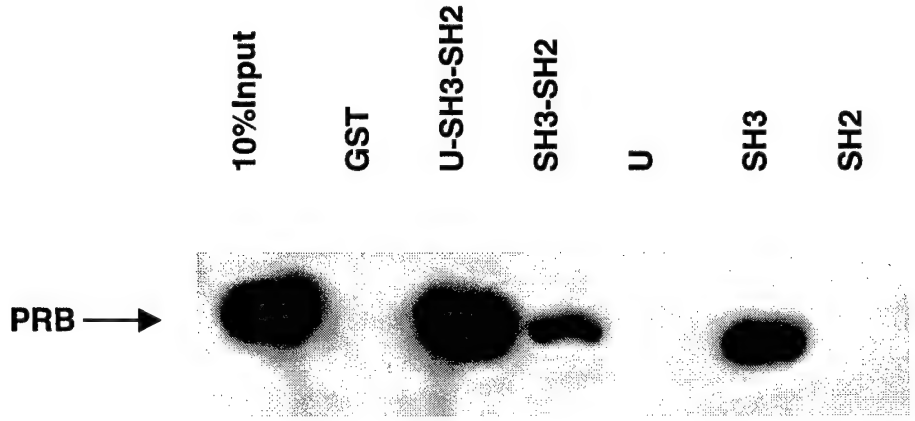


Figure 4. PR interacts in vitro with SH3 domains of other proteins including Src family kinases. PR-B (upper panel) or the proline mutant PR-B(mPro) (lower panel) were expressed in the baculovirus system bound to R5020 and were incubated with either GST or the different GST-SH3 domain fusion proteins indicated immobilized to Glutathione Sepharose. Bound PR was analyzed by immunoblot assay with PR specific MAb PR1294 and the left most lane in each panel represents 10% assay input of PR-B and PR-B(mPro). As determined by immunoblot assay with a GST specific MAb (794/H12) free GST and each of the GST-fusion proteins were immobilized in approximately equal amounts (not shown).

A


SH2-kinase
linker
Myr
U
SH3
SH2
PXPP
Kinase
Y
527
Y
416

B


10%Input
GST
U-SH3-SH2
SH3-SH2
U
SH3
SH2
PRB

C

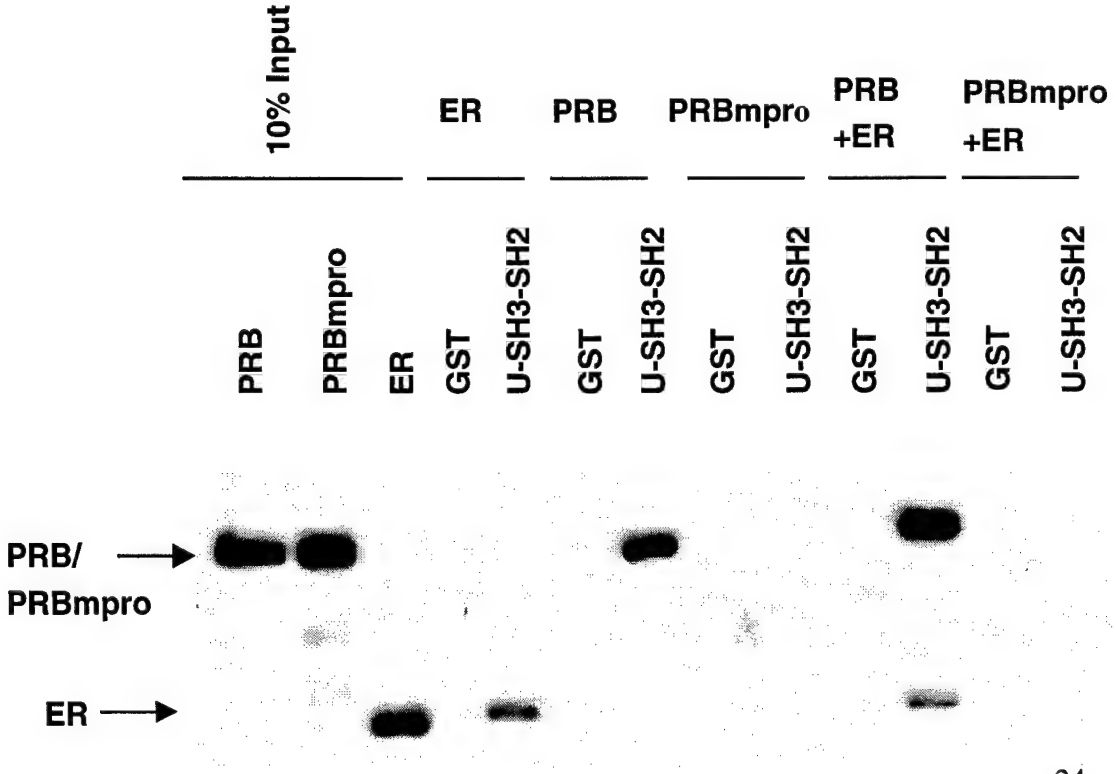

10% Input
ER
PRB
PRBmpro
PRB
+ER
PRBmpro
+ER
PRB
PRBmpro
ER
GST
U-SH3-SH2
GST
U-SH3-SH2
GST
U-SH3-SH2
GST
U-SH3-SH2
GST
U-SH3-SH2
PRB/
PRBmpro
ER

Figure 5 Direct binding of ER and PR to c-Src domains. A) Schematic of c-Src. Myr, N-terminal myristeration site; U, N-terminal unique region; PXPP, polyproline sequence in SH2-kinase linker region; Kinase, c-terminal catalytic domain. **B)** Purified baculovirus expressed 6X histidine-tagged liganded- human PRB were incubated with either GST or GST fusion c-Src domains indicated immobilized to Glutathione Sepharose. Bound PR was analyzed by immunoblot assay with PR specific Mab 1294. First lane represent 10% assy input of purified PR-B **C)** Purified baculovirus expressed 6X histidine-tagged liganded-human PRB, PRB_{mpro} ,ER, or the combination of PR+ER or PR_{mpro} +ER incubated with GST-U-SH3-SH2 domains of c-Src immobilized to Glutathione Sepharose beads. Bound receptors were eluted and analyzed by immunoblotting with Mab specific for 6X polyhistidine tag.

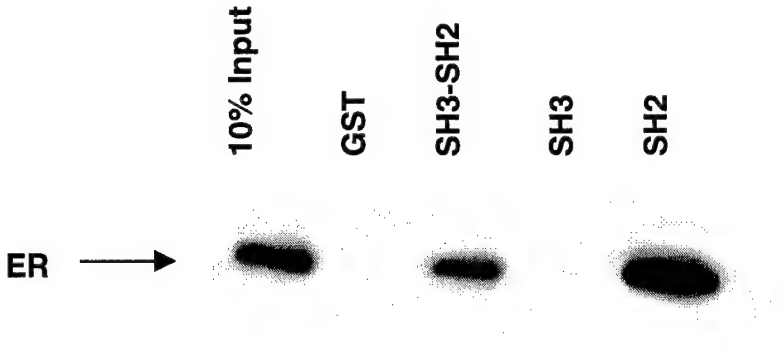


Figure 6 Direct binding of ER to c-Src SH2 domain. Purified baculovirus expressed human ER bound to estradiol was incubated with GST or GST fusion of different c-Src domains indicated immobilized to Glutathione Sepharose. Bound ER was analyzed by immunoblotting with ER specific Mab and the first lane represent 10% of assay input of purified human ER. As determined by immunoblot with a GST specific Mab(794/H12) free GST and each of the GST-fusion c-Src domain were immobilized in approximately equal amount (not shown)

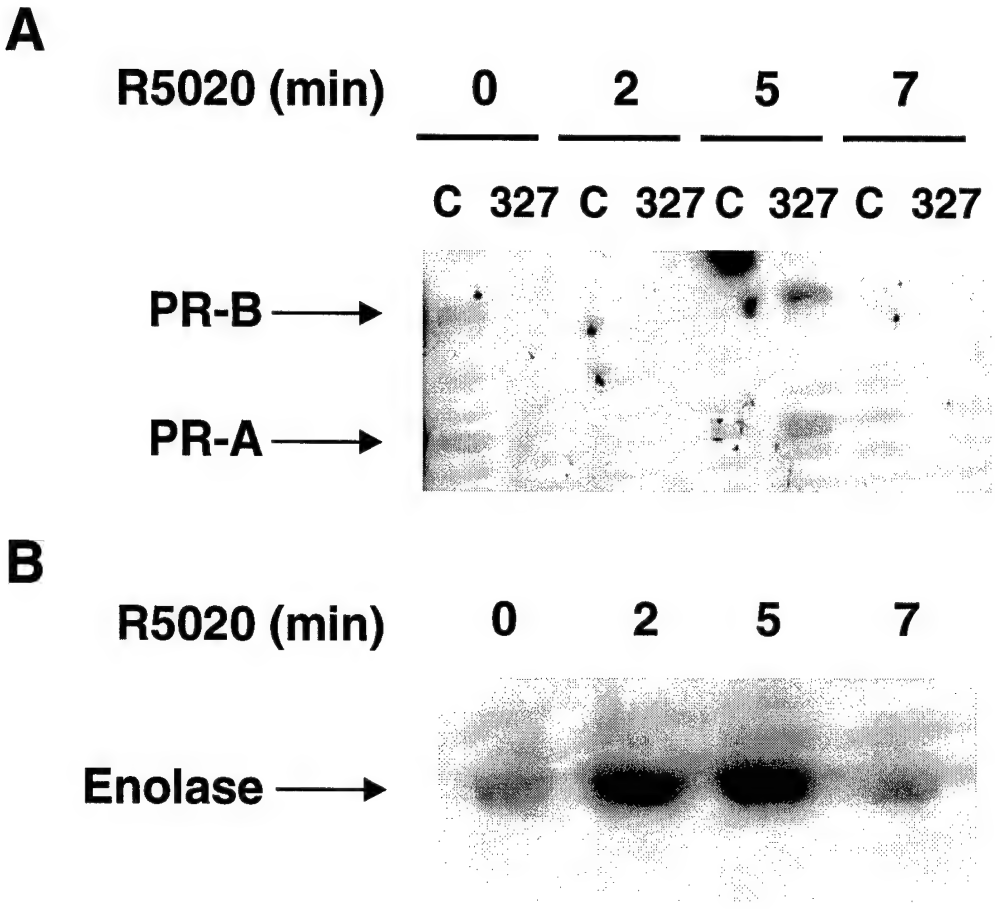
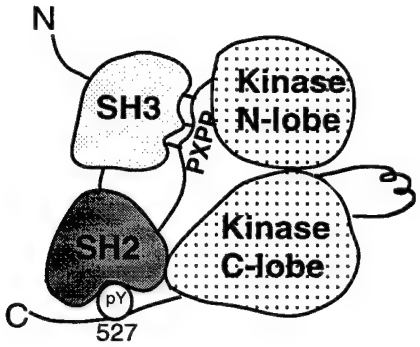

A
R5020 (min)
0
2
5
7
C 327 C 327 C 327 C 327
PR-B
PR-A
B
R5020 (min)
0
2
5
7
Enolase
C
PRB+ER
PRBmpro+ER
R5020 (min)
0
5
0
5
Enolase

Figure 7 PR interaction with c-Src in whole cells is hormone dependent and R5020 treatment enhances Src-kinase activity. T47D breast cancer cells were grown in phenol red free medium containing 5% charcoal-dextran treated fetal calf serum (DCC-FBS) for seven days. Cells were treated with 10nM R5020 for 2, 5, and 7 min and lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 4 mM EDTA, 30 mM KCl, 1 mM PMSF, 1 μg each of aprotinin, leupeptin, pepstatin and bacitracin, 1 mM Na orthovanadate and 1% NP-40), and centrifuged at 15000 g for 15 min. Protein concentration for clear supernatant was assayed. Cell lysates were diluted to a protein concentration of 1 mg/ml and incubated with 1 μg of c-Src MAb 327 Ab for 90 min at 4 C. The antibodies were absorbed by addition of a 50% suspension of protein G-Sepharose and incubate for additional 30 min. The samples were centrifuged and pellets were washed with lysis buffer three times. **A)** Bound proteins were eluted and analyzed by immunoblotting with PR specific MAb 1294 Ab. **B)** Immunoprecipitate pellets were assayed for c-Src kinase activities using acid modified enolase as substrate. **C)** Cos-7 cells were transfected with expression vector for human ER, c-Src along with wild type PR-B or PRB_{mpro}. Cells were treated with 10 nM R5020 for 5 min and lysed with lysis buffer, immunoprecipitated with 327 Ab and assayed for c-Src activity as described above.

A


N
SH3
Kinase
N-lobe
PXPP
SH2
Kinase
C-lobe
pY
C
527

B

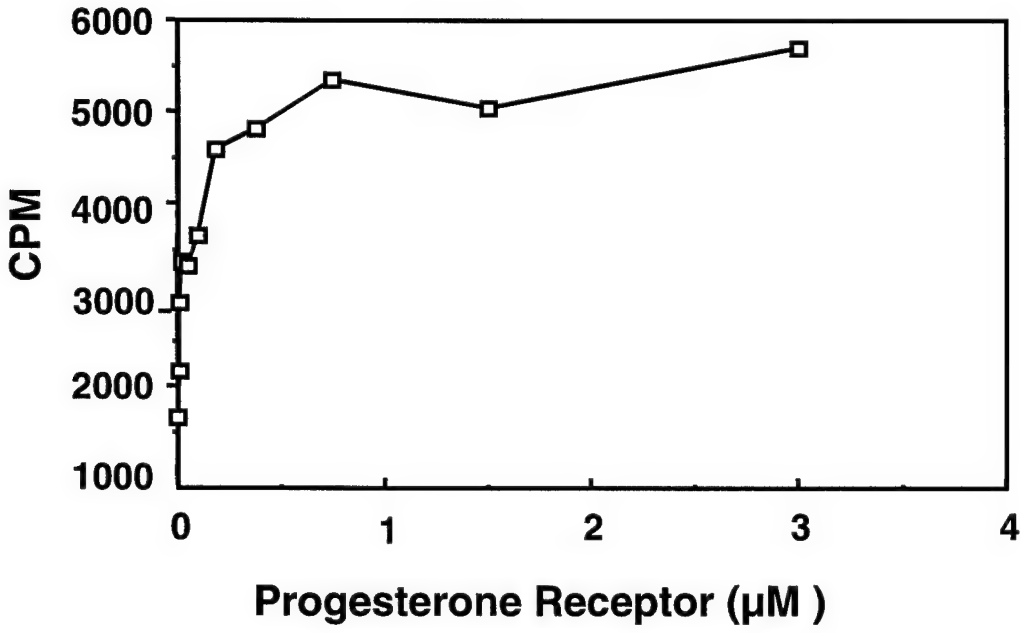

6000
5000
4000
3000
2000
1000
CPM
0
1
2
3
4
Progesterone Receptor (μM)

Figure 8 Down regulated HcK is activated by PR *in vitro*. A) Schematic of c-Src in a closed conformation by SH3 association with a proline rich sequence in the SH2-kinase linker and SH2 association with phosphotyrosine at position 527. **B)** Down regulated Hck was incubated with varying concentrations (6 nM to 3 μM) of purified wild type PR-B bound to hormone (R5020). Hck enzymatic activity was measured as CPM of 32-ATP incorporated into a synthetic peptide substrate

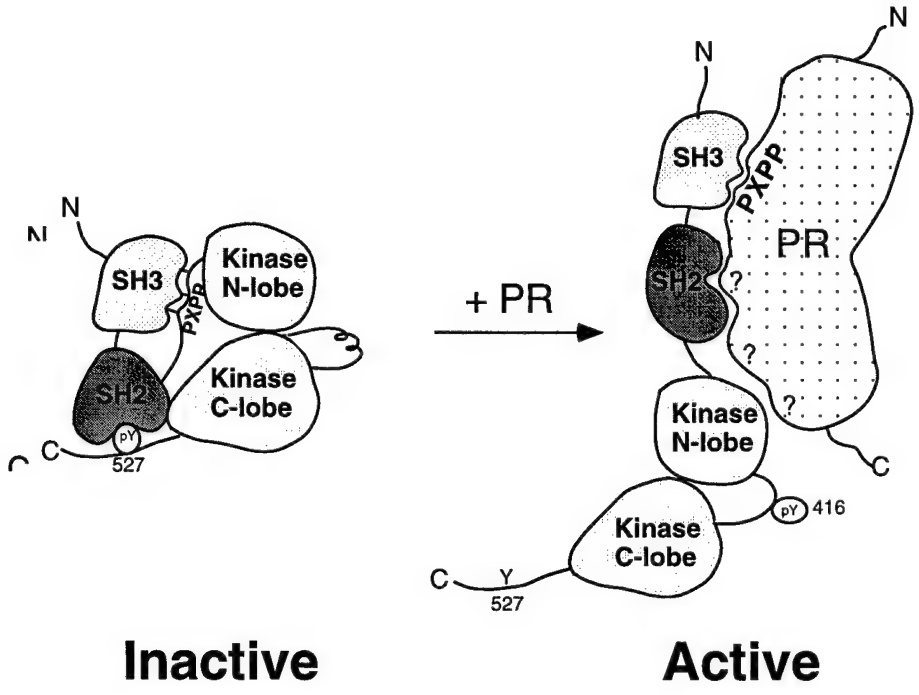


Figure 9 Proposed model for PR activation of Src kinase activity by domain displacement. The associations engaged by SH3 and SH2 of Src kinase keeping the kinase in a closed-inactive conformation. Displacement of the interaction between SH2-kinase linker region (PXXP) and SH3 with a proline rich PPII sequence present in the N-terminus of PR causes the kinase to open up and assume an open-active conformation

References

1. **Berthois, Y., N. Pourreau-Schneider, P. Gaudilhon, H. Mittre, N. Tubiana, and P. M. Martin** 1986. Estradiol membrane binding sites on human breast cancer cell lines. Use of fluorescent estradiol conjugate to demonstrate plasma membrane binding system J Steroid Biochem. **25:**963-972.
2. **Boonyaratnakornkit, V., V. Melvin, P. Prendergast, M. Altman, L. Ronfani, M. E. Bianchi, L. Taraseviciene, S. K. Nordeen, E. A. Allergretto, and D. P. Edwards** 1998. High-mobility group chromatin protein 1 and 2 functionally interact with steroid hormone receptors to enhace their DNA binding in vitro and transcriptional activity in mammalian cells Mol Cell Biol. **18:**4471-4487.
3. **Briggs, S. D., and T. E. Smithgall** 1999. SH2-kinase linker mutations release Hck tyrosine kinase and transforming activities in Rat-2 fibroblasts J Biol Chem. **274:**26579-26583.
4. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Granziani, R. Kappeller, and S. Soltoff** 1991. Oncogenes and signal transduction Cell. **64:**281-302.
5. **Castoria, G., M. V. Barone, M. D. Demenico, A. Bilancio, D. Ametrano, A. Migliaccio, and F. Auricchio** 1999. Non-transcriptional action of estradiol and progestin triggers DNA synthesis EMBO. **18:**2500-2510.
6. **Downward, J.** 1997. Cell cycle: routine role for Ras Curr Biol. **7:**258-260.
7. **Eliceiri, B. P., R. Paul, P. L. Schwartzbreg, J. D. Hood, J. Leng, and D. A. Cheresh** 1999. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability Mol Cell. **4:**915-924.
8. **Guy, C. T., S. K. Muthuswamy, R. D. Cardiff, P. Soriano, and W. J. Muller** 1994. Activation of the c-src kinase is required for the induction of mammary tumors in transgenic mice Genes Dev. **8:**23-32.
9. **Hunter, T.** 2000. Signaling-2000 and beyond Cell. **100:**113-127.
10. **Kastner, P., A. Krust, B. Turcotte, U. Stropp, L. Tora, H. Gronemeyer, and P. Chambon** 1990. Two distinct estrogen-regulated promoters generate transcripts encoding two functionally different human progesterone receptor forms A and B EMBO J. **9:**1603-1614.
11. **Leslie, K. K., N. S. Kumar, and K. Fox** 2000. A novel mechanism underlying progestin resistance in endometrial cancer: loss of the nuclear localization of progesterone B receptor, p. 105. Keystone Symposia: Nuclear receptor 2000, Steamboat Springs, CO.
12. **Marshall, C. J.** 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase Curr Opin Genet Dev. **4:**82-89.

13. **McDonnell, D. P., and M. E. Goldman** 1994. RU 486 exerts antiestrogenic activities through a novel progesterone receptor A form-mediated mechanism J Biol Chem. **269:**1945-1949.
14. **Migliaccio, A., M. D. Domenico, G. Castoria, A. Falco, P. Bontempo, E. Nola, and F. Auricchio** 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells EMBO. **15:**1292-1300.
15. **Migliaccio, A., D. Piccolo, G. Castoria, M. D. Demenico, A. Bilancio, M. Lombardi, W. Gong, M. Beato, and F. Auricchio** 1998. Activation of Src/p21ras/Erk pathway by progesterone via cross-talk with estrogen receptor EMBO. **17:**2008-2018.
16. **Moarefi, I., M. LaFevre-Bernt, F. Sicheri, M. Huse, C.-H. Lee, J. Kuriyan, and W. T. Miller** 1997. Activation of the Src-family tyrosine kinase by SH3 domain displacement Nature. **385:**650-653.
17. **Nguyen, J. T., C. W. Truck, F. E. Cohen, R. N. Zucherman, and W. A. Lim** 1998. Expoliting the basis of proline recognition by SH3 and WW domains: designs of N-substituted inhibitors Nature. **282:**2088-2091.
18. **Ottenhoff-Kalff, A. E., G. Riijksen, E. A. van Beurden, A. Hennipman, A. A. Michels, and G. E. J. Staal** 1992. Characterization of protein tyrosine kinases from human breast cancer involvement of the c-src oncogene product Cancer Res. **52:**4473-4778.
19. **Papas, T. C., B. Gametchu, and C. S. Watson** 1995. Membrane estrogen receptor identified by multiple labeling and impeded-ligand binding FASEB J. **9:**404-410.
20. **Pawson, T.** 1997. New impression of Src and Hck Nature. **385:**582-585.
21. **Pellicena, P., K. R. Stowell, and W. T. Miller** 1998. Enhanced phophorylation of Src kinase family substrates containing SH2 domain binding sites J Biol Chem. **273:**15325-15328.
22. **Porter, M., T. Schindler, J. Kuriyan, and W. T. Miller** 2000. Reciprocal regulation of Hck activity by phosphorylation of Tyr527 and Tyr416 J Biol Chem. **275:**2721-2726.
23. **Revelli, A., M. Massobrio, and J. Tesasik** 1998. Nongenomic actions of steroid hormones in reproductive tissues Endocrine Rev. **19:**3-17.
24. **Ribon, V., R. Herrera, B. K. Kay, and A. R. Saltiel** 1998. A role for CAP, a novel multifunctional Src homology 3 domain-containing protein in formation of actin stress fibers and focal adhesions J Biol Chem. **273:**4073-4080.
25. **Ribon, V., J. A. Printen, N. G. Hoffman, B. K. Kay, and A. R. Saltiel** 1998. A novel, mutifunctional c-Cbl binding protein in insulin receptor signaling in 3T3-L1 adipocytes Mol Cell Biol. **18:**872-879.
26. **Rosen, N., J. Bolen, A. M. Schwartz, P. Cohen, V. De Seau, and M. A. Israel** 1986. Analysis of pp60 c-src protein kinase activity in human tumor and tissues.

27. **Satorius, C. A., S. D. Groshong, R. A. Miller, R. L. Powell, L. Tung, G. S. Takimoto, and K. B. Horwitz** 1994. New T47D breast cancer cells lines for the independent study of progesterone B and A receptors; only antiprogestin-occupied B receptor are switched to transcriptional agonists by cAMP Cancer Res. **54:**3868-3877.

28. **Sicheri, F., I. Moarefi, and J. Kuriyan** 1997. Crystal structure of the Src-family tyrosine kinase Hck Nature. **385:**602-609.

29. **Silberstein, G. B., K. Van Horn, G. Shyamala, and C. W. Daniel** 1996. Progesterone receptor in the mouse mammary duct: distribution and developemental regulation Cell Growth & Differentiation. **72:**945-952.

30. **Tung, L., M. K. Mohamed, J. P. Hoeffler, G. S. Takimoto, and K. B. Horwitz** 1993. Antagonist-occupied human progesterone B-receptors activate transcription without binding to progestorne response elements and are dominantly inhibited by A-receptors. Molecular Endocrinol. **7:**1256-1265.

31. **Vegato, E., M. M. Shahbaz, D. X. Wen, M. E. Goldman, B. W. O'Malley, and D. P. McDonnell** 1993. Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. Molecular Endocrinol. **7:**1244-1255.

32. **Wong, B. R., D. Besser, N. Kim, J. R. Arron, M. Vologodskaia, H. Hanafusa, and Y. Choi** 1999. TRANCE, a TNF family member activates Akt/PKB through a signalling complex involving TRAF6 and c-Src Mol Cell. **4:**1041-1049.

33. **Xu, W., A. Doshi, M. Lei, M. J. Eck, and S. C. Harrison** 1999. Crystal structures of c-Src reveal features of its autoinhibitory mechanism Mol Cell. **3:**629-638.

34. **Xu, W., S. C. Harrison, and M. J. Eck** 1997. Three-dimensional structure of the tyrosine kinase c-Src Nature. **385:**595-602.

35. **Yuan, Z.-M., H. Shioya, T. Ishiko, X. Sun, J. Gu, H. YinYin, H. Lu, S. Kharbanda, R. Weichselbaum, and D. Kufe** 1999. p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage Nature. **399:**814-817.

Appendices

Abstract presented at Keystone Symposia Nuclear Receptors 2000, March 25-31,2000, Stemboat Springs, Colorado. Abstract #208

Progesterone receptor contains a proline rich sequence in the N domain that directly interacts with SH3 domains and activates Src- tyrosine kinases.

V. Boonyaratanakornkit[1], M. Porter, S.A. Anderson[1], W.T. Miller[2], D.P. Edwards[1], Pathology, Univ. of Colo Hlth Sci Ctr, Denver, CO 80262, Physiology&Biophysics, SUNY, Stony Brook, NY 11794.

Using a yeast two hybrid screen to identify proteins that interact with and mediate functions of the N- terminus of human progesterone receptor (PR), a clone was isolated that encodes for a protein with significant homology to the SH3 domain the c-Cbl-associated protein, termed CAP. PR interacts with CAPSH3 *in vivo* in a hormone dependent manner by yeast and mammalian two hybrid assays. By GST-pull down assay, PR interacted directly with CAPSH3, however, the interaction was not hormone-dependent. The N-domain of PR was discovered to contain a proline rich sequence (aa 421-428) that conforms to a consensus class II polyproline helix (PPII) ligand for SH3 domains and is required for PR interaction. Mutation of three proline residues to alanine abolished PR-CAPSH3 interaction. Based on sequences, no other nuclear hormone receptor contains a predicted PP helix and SH3 interactions were not detected with several other nuclear receptors, suggesting this is a unique property of PR. In addition, PR interacted efficiently *in vitro* with selected SH3 domains of other proteins including the Src-family tyrosine kinases (Hck and Src) and these interactions also were dependent on the PPII sequence in PR. PR and Src also interacted within mammalian cells as detected by coimmunoprecipitation assays of cells that express both proteins. The Src-family kinases are autoinhibited by intramolecular associations between the SH2 domain and a C-terminal tyrosine phosphorylation sites and between the SH3 domain and a polyproline like helix in the linker connecting the catalytic domain, keeping the kinase in closed inactive conformation. Disruption by dephosphorylation and/or competition by external ligands convert the kinase to an active open conformation. *In vitro*, PR was a potent activator of tyrosine phosphorylated down regulated Hck via displacement of the intramolecular association of SH3 domain with the catalytic domain; the proline mutant PR had no influence on Hck activity. Mutations in the PPII did not influence the ability of PR to function as a transcriptional activator of classical target gene promoters. These results suggest a novel functional role for progesterone and PR as activators of Src-kinase signaling pathways through direct interactions with SH3 domains and may begin to define a mechanism for non-genomic actions of progesterone at cell or nuclear membranes.

Abstract for The Endocrine Society's 82nd Annual Meeting,. ,June 21-24, 2000, Toronto, Canada. Abstract # 1971

Progesterone receptor contains proline-rich sequences that directly interact with SH3 domains of Src-tyrosine kinase family members. V. Boonyaratanakornkit[1], M.P. Scott[2], S.M. Anderson[1], W.T. Miller[2], and D.P. Edwards[1], Pathology Dept and Mol. Bio. Prog , U of Colorado HSC, Denver, CO 80262[1]. Dept of Physiology and Biophysics, SUNY, Stony Brook, NY 11794.

As sequence specific transcriptional activators, progesterone (PR) and estrogen receptor (ER) mediate many of the biological actions of progesterone and estrogen through direct activation of target genes. There is increasing evidence that PR and ER can also mediate non-genomic effects through interaction with and activation of the Src/Ras/Erk signaling pathway. Using full length PR (A-form) as the target in a yeast two hybrid screen, the SH3 domain of c-Cbl-associated protein (CAP) was identified to interact with the N-terminal domain of PR in a hormone dependent manner *in vivo*. By GST-pull down experiments, PR was found to interact directly *in vitro* with the SH3 domain of CAP as well as selected SH3 domains of other signaling molecules including the Src-tyrosine kinase family members: c-Src and Hck. The N-domain of PR contains a proline-rich sequence (aa 421-428) that conforms to a consensus class II polyproline helix (PPII) ligand for SH3 domains and was shown by mutagenesis to be required for PR interaction with SH3 domains. Other nuclear receptors lack the PPII sequence and those tested (ER, GR, AR and TR) failed to interact with SH3 domains *in vitro*. PR interaction with full length Src and Hck involved additional hormone-dependent determinants in the ligand binding domain (LBD) of PR and unknown kinase determinants outside of the SH3 domain. The interaction between PR and Src *in vitro* was enhanced by addition of ER and a tertiary complex between PR-Src and ER was also detected. The interaction between ER and Src was mapped to the SH2 domain. Using coimmunoprecipation, a transient hormone-dependent interaction between PR and Src was detected in mammalian cells that was optimal at 5 min of treatment. The Src-family kinases are autoinhibited by intramolecular associations between the SH2 domain and a C-terminal tyrosine phosphorylation site and between the SH3 domain and a polyproline like helix in the linker connecting the catalytic domain keeping the kinase in an inactive conformation. Disruption of these interactions by dephosphorylation and/or competition by external ligands converts the kinase to an active conformation. *In vitro*, PR was a potent activator of tyrosine phosphorylated down regulated Hck via displacement of the intramolecular association of SH3 domain with the catalytic domain. These results suggest a novel function role for PR as an activator of Src-kinase signaling pathways through direct interaction with the SH3 domain and a possible role of ER which enhances Src-PR interaction. Together, these findings may begin to unravel the molecular mechanism for non-genomic actions of estrogen and progesterone

THE ENDOCRINE SOCIETY

4350 EAST WEST HIGHWAY
SUITE 500
BETHESDA, MARYLAND
20814-4426

TELEPHONE 301.941.0200
FAX 301.941.0259

www.endo-society.org

March 20, 2000

Viroj Boonyaratanakornkit
Pathology
University of Colorado Health Sciences Ctr
Campus Box B-216
4200 E. 9th Avenue
Denver, CO 80262

Dear Dr. Boonyaratanakornkit:

Congratulations! You have been selected by the Annual Meeting Steering Committee as one of five recipients of the 2000 Burroughs Wellcome Fund Travel Award for your abstract #1971 entitled, "PROGESTERONE RECEPTOR CONTAINS PROLINE-RICH SEQUENCES THAT DIRECTLY INTERACT WITH SH3 DOMAINS OF SRC-TYROSINE KINASE FAMILY MEMBERS." The Award, in the amount of $1,000 must be used in conjunction with attendance and presentation of this abstract at ENDO 2000, June 21-24, 2000, in Toronto, Canada. You will receive the date and time of your abstract presentation under separate cover.

Your award certificate will be presented during the Student/Fellow Reception on Tuesday, June 20 at 6:00 p.m. Please note that this is the evening before the meeting begins, so please plan to arrive in time to attend the reception.

In the meantime, please provide me with the following:

1. **Completed W-9 form.** You must complete the enclosed W-9 form and return it to my attention. The honorarium check will be issued upon its receipt. If you are not subject to U.S. taxes, please complete the name and address portion and write "not applicable" in the "Taxpayer Identification Number" space.

2. **Name and fax number of the public affairs officer at your institution.** We will be preparing a press release on your award. We will also mail you a copy of the announcement for distribution to any local media.

3. **Your name and degree(s)** as it should appear on the award certificate.

Feel free to contact me if you have any questions at 301-941-0211 or e-mail: tjacobson@endo-society.org. I look forward to seeing you in Toronto.

Best regards,

Terry C. Jacobson

Terry Cecilia Jacobson
Manager, Professional Affairs

Enclosure: W-9 Form